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Journal of Chromatography B, 784 (2003) 33–38
JOURNAL OF CHROMATOGRAPHY B
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One step purification of alpha$_1$-acid glycoprotein from human plasma
Fractionation of its polymorphic allele products

Federica Azzimonti[a], Daniel H. Atchley[a], Catherine Anne Morrison[a], Seetal Dodd[b,1], David W. Boulton[b,2], C. Lindsay DeVane[b], Philippe Arnaud[a,b,*]

[a]*Department of Microbiology and Immunology, Medical University of South Carolina, Charleston, SC 29425, USA*
[b]*Laboratory of Drug Disposition and Pharmacogenetics, Department of Psychiatry and Behavioral Sciences, Medical University of South Carolina, Charleston, SC 29425, USA*

Received 11 October 2001; received in revised form 2 July 2002; accepted 20 September 2002

Abstract

Alpha$_1$-acid glycoprotein is a plasma protein that exhibits both microheterogeneity and polymorphism. Its purification from human plasma is usually performed using a sequence of different fractionation steps. Here we report a one-step isolation technique of this protein based upon pseudo-ligand affinity chromatography on immobilized Cibacron Blue F3GA at acidic pH. In addition, the use of two narrow pH elution buffers allows us to separate the two genetic products of this protein, which differ from each other by 21 amino acid substitutions. This technique will facilitate the study of the structural, biological and pharmacokinetic properties of each individual allele product.

Keywords: α_1-Acid glycoprotein; Polymorphic allele products

1. Introduction

Alpha$_1$-acid glycoprotein, also called orosomucoid (ORM) is a glycoprotein synthesized mostly by hepatocytes [1]. It is present in healthy human plasma at concentrations between 0.55 and 1.4 mg/ml [2]. ORM is an acute-phase reactant protein, and its concentration increases up to four times its basal levels following infection and/or inflammation [3]. It represents the major component of the seromucoid proteins fraction, from where it was first isolated [4].

The ORM protein present in plasma is heterogeneous, and its primary sequence shows amino acid (aa) substitutions in several positions of the molecule [5]. This is due to the fact that ORM represents a mixture of at least two proteins. This was demonstrated when the ORM gene was found to be duplicated on each chromosome with each locus (ORMA and ORMB) coding for a different protein [6,7]. Ideas on the relative expression of each protein

*Corresponding author. Tel.: +1-843-792-4349; fax: +1-843-792-2464.
E-mail address: arnaudp@musc.edu (P. Arnaud).
[1]Present address: Victorian College of Pharmacy, Melbourne, Victoria, Australia.
[2]Present address: Bristol–Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, USA.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00749-3

have evolved; indeed, original work tended to indicate that the contribution of the ORM2 locus represented about 1% of the total ORM values, and that its expression was not affected by the acute phase phenomenon [7]. In fact, later work showed that the second locus contributed 30 to 40% of the total ORM concentration, and that its increase following acute phase was similar to that of ORM1 [8–10]. Determination of their respective values relies upon the separation of the two isoforms by isoelectric focusing following desialylation, followed by Western blotting and quantitation of the bands by densitometry. The fact that the isoproteins may react differently with commercial polyclonal antibodies [11] is an additional factor which renders the quantitation of the isoproteins difficult.

A number of biological properties have been assigned to ORM over the years. Its role as an immunomodulator has been extensively reported [12–17] and appears to involve the glycan part of the molecule. Recent work indicates that ORM possesses an anti-apoptotic effect [18] and that it plays an important role in the permselectivity of capillary walls [19].

Most importantly, ORM is known to be an important carrier for basic drugs in plasma, affecting their volume of distribution. The importance of its concentration on the determination of the free fraction of these drugs has been largely demonstrated [20,21]. This binding is complex, as some drugs apparently bind similarly to each allele product while several others bind predominantly, even exclusively, to one of the variants of ORM [22–26]. In this respect, it is important to be able to separately purify each allele product for pharmacogenetic studies, in particular when a new drug is introduced on the market. The present article reports a one-step technique to achieve this purpose.

2. Experimental

2.1. Samples

Venous blood was obtained from healthy volunteers presenting the three common phenotypes (ORM1*F1, ORM2 *A; ORM1*S, ORM2*A and ORM1*F1S, ORM2*A) with informed consent and collected into heparinized containers. Plasma was collected following centrifugation, aliquoted and frozen at −20 °C until used.

In some experiments, purified ORM, obtained from Sigma (Ref. G 9885), was diluted at a concentration of 10 mg/ml in the starting buffer and applied to the columns.

2.2. Column chromatography

Cibacron Blue F3GA (1.9 mg dye/ml) attached to agarose (AffiGel Blue Gel from BioRad, Hercules, CA, 100–200 mesh) was poured in a FPLC column (Amersham–Pharmacia Biotech, Piscataway, NJ, 100×10 mm), which contained 14 ml of gel equilibrated in the starting buffer. This column was used with a series of buffers in preliminary experiments to determine the best elution conditions. The sample (1.0 ml) dialyzed overnight at 4 °C against 1 l of starting buffer, was applied using a superloop. The flow-rate was 1.0 ml/min, and 1.0 ml fractions were collected. Once the optimal conditions were determined, the method was scaled up by using a larger column (200×25 mm column, containing 80 ml of immobilized dye) equipped with plungers, and 3.0 ml samples were applied. The flow-rate was 2.0 ml/min. The columns were coupled to a FPLC system (Amersham–Pharmacia) which allowed the entire automation of the chromatographic steps.

Acetate and phosphate buffers (0.03 *M*) of different pHs were used to optimize the separation of the proteins, and the optical density of the elutes was monitored at 280 nm. Following the collection of the fractions of interest, which were concentrated to the original volume of the sample applied (1.0 and 3.0 ml, respectively) by centrifugation using Vivaspin 10 K MWCO from Sartorius, the columns were stripped with 0.5 *M* sodium thiocyanate and re-equilibrated with the starting buffers.

2.3. Analysis of ORM

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% gels according to standard procedures, using a mini-gel apparatus (BioRad). Gels were either stained with Coomassie Blue or transblotted for 2 h at 200 mA constant current using a Tris–glycine (25 m*M* Tris,

192 m*M* glycine, pH 8.3) buffer in a transblot apparatus (BioRad). Nitrocellulose membranes were obtained from Schleicher & Schuell, Keene, NH (BA 85, 0.45 μm) and positioned on the anodal side of the gel.

Following the electrotransfer, the membranes were stained with the two antibody technique, using a primary antibody (sheep anti-ORM) obtained from The Binding Site, San Diego, CA, and diluted at 1/1000, and a secondary (goat anti-sheep) antibody peroxidase-labeled obtained from Pierce, Rockford, IL, and diluted to 1/8000. The membranes were revealed by chemoluminescence, using the Super-Signal West substrate provided by Pierce. The films were scanned on a Microtek Scanmaker III analyzer (Microtek, Taiwan).

Isoelectric focusing (IEF) was performed in immobilized pH gradient (IEF–IPG) gels obtained from Amersham–Pharmacia, according to the technique originally reported by Eap and Baumann [27]. The IEF–IPG gels (pH 4.5–5.4) were rehydrated for 2 h with a solution containing 8.0 *M* urea (Fluka GMBH), 2% 2-mercaptoethanol and 8.7% glycerol. Samples applied to the gel were first desialylated. Briefly, 10 μl plasma samples were diluted at a 1/3 ratio in a 5 m*M* acetate buffer (pH 5.5) containing 15.4 m*M* NaCl and 0.9 m*M* $CaCl_2$ and one unit/ml of *C. perfringens* neuraminidase (Sigma, St Louis, MO, type V), and incubated at 37 °C overnight. Samples (20 μl) were applied on the gel using Whatman 3 MM paper tabs. The gels were run first at 500 V, 5 mA and 5 W for 1 h, then the papers were removed and the gels were run overnight at 2000 V, 5 W, 5 mA. The gels were either stained with Coomassie Blue, or submitted to pressure blotting for 2 h at room temperature using the above membranes (Schleicher & Schuell). Immunostaining of the membranes was performed with the two-antibody technique and revealed as above. In some cases, native or asialo-ORM were examined on thin (0.2 mm) polyacrylamide gels ($T=30$, $C=4$) made with a final concentration of 2% (w/v) 2.5–4 (2/3) and 4–6 (1/3) carrier ampholytes (Amersham–Pharmacia) and polymerized with TEMED and ammonium persulfate [37].

Protein quantitation was performed by radial immunodiffusion, using plates and standards from The Binding Site. The concentration of purified proteins was further estimated by absorbance at 280 nm, using an extinction coefficient of 8.9 [28].

3. Results

Preliminary experiments indicated that, when plasma dialyzed at pH 4.5 was run on the Blue column equilibrated also at pH 4.5 and eluted with the same buffer, a single protein peak was eluted [3]. Analysis by SDS–PAGE and immunoblotting indicated that the single component present in this peak was ORM. Nevertheless, some additional ORM was eluted when the pH was raised to 5.6, together with other proteins including $alpha_1$-antitrypsin and $alpha_2$-HS glycoprotein, as evidenced by Western blotting and IEF. Examination of both fractions by IEF–IPG indicated that the first peak included ORM1 and ORM2 proteins when the second peak contained only ORM1 (results not shown).

Equilibration of the column and the sample at pH 4.0 and elution of the column with a linear pH gradient between 4.0 and 5.6 showed that ORM2 would elute from the column at a pH of 4.0, while ORM1 was eluted at a slightly more basic pH, i.e. 4.95. Knowing these values, it was then possible to elute both proteins separately (Fig. 1), with no apparent cross-contamination and with no detectable elution of other plasma proteins, as shown by the presence of a unique ORM band on SDS–PAGE gels (Fig. 2). Confirmation that ORM2 was indeed separated from ORM1 was evidenced on Western blot after IEF–IPG of desialylated peaks 1 and 2 (Fig. 3). Similarly, ORM2 was separated from ORM1 when (apparent) homozygous plasma samples for ORM1 (ORM1F1 and ORM1S) were applied on the column (not shown).

The yield of the purification step, measured from comparison between the values of ORM in the starting material and the sum of each fraction, was consistently higher than 80%. In addition, no ORM could be detected by Western blotting following SDS–PAGE of the proteins found in the thiocyanate eluate. The apparent absence of denaturation, postulated on the results of IEF–IPG, which showed a pI of the asialo-proteins identical to that of the starting material (see Fig. 3), was confirmed by comparison of the non-desialylated proteins on SDS–PAGE

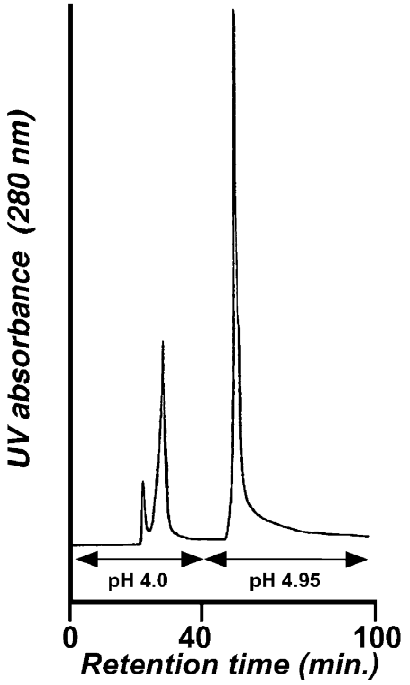

Fig. 1. Elution profile (OD 280 nm) of human plasma (3.0 m, dialyzed against the starting buffer) applied to a Cibacron Blue F3GA column. Following sample application, the column, equilibrated with 0.03 *M* sodium acetate, pH 4.0, was developed with the same buffer. After elution of the first peak, the pH of the buffer was increased to 4.95. Note the presence of a small peak just before peak 1, which was consistently observed and corresponded to the same protein found in peak 1 (ORM2), as shown on Fig. 3, lane 2. The flow-rate of the buffers was 2 ml/min.

(regular IEF gels at acidic pH (results not shown)). Finally, quantitation by IEF–IPG of the respective amount of the ORM proteins purified indicated that each protein was present after purification in the same ratio as that of the starting material

4. Discussion

As indicated above, amino acid sequence studies showed that ORM presented substitutions at 21 positions out of a total of 181 residues [5]. Reasons for this heterogeneity were solved when Dente et al. determined the cDNA sequence of ORM and demonstrated that the cDNA they isolated corresponded indeed to only one of the two sequences reported by Schmid et al. [6]. This indicated that the ORM protein sequenced earlier was a mixture of at least

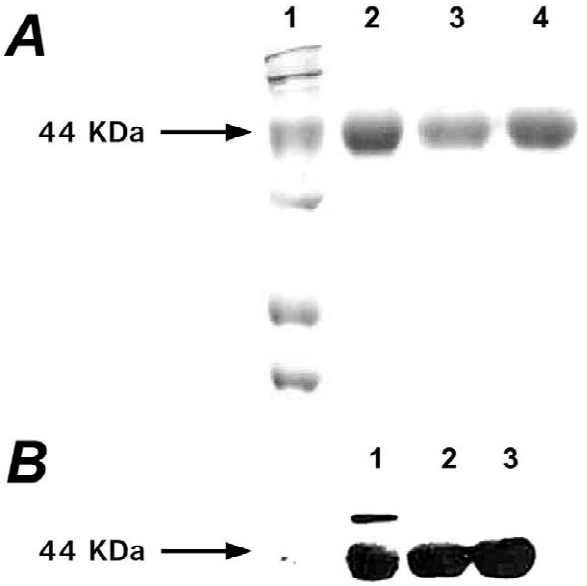

Fig. 2. SDS–polyacrylamide (12%) gel electrophoresis of ORM fractions following pseudo-ligand affinity chromatography on Cibacron Blue. (A) Gel stained for proteins (Coomassie Blue R). Lane 1: MW markers (from Biorad); Lane 2: ORM control; Lane 3: peak 1, eluted at pH 4.0; Lane 4: Peak 2, eluted at pH 4.95. (B) Western blot of the fractions described above. The gel was blotted onto a nitrocellulose membrane which was revealed with the two-antibody technique (sheep anti-ORM antibody, then goat anti-sheep peroxidase-labeled antibody) and revealed by enhanced chemoluminescence. Lane 1: ORM control (purified by preparative IEF, see Ref. [33]). Lane 2: Peak 1; Lane 3: Peak 2; The small band visible in the control ORM with a M_r approximately twice that of ORM, probably corresponds to a dimer of the protein (see Ref. [51]).

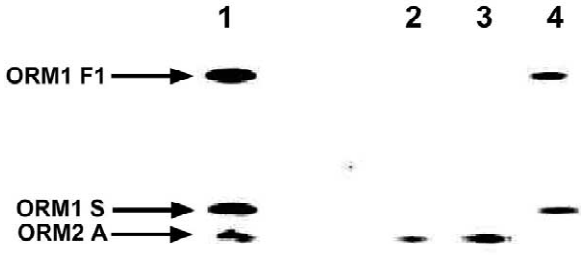

Fig. 3. Isoelectric focusing–immunoblot analysis of the ORM isoproteins fractionated on the Cibacron Blue column as indicated above. Anode at the top. Following desialylation, the plasma and aliquots from the fractions eluted were run on an IPG gel (pH 4.5–5.4). After completion of the run, the gel was blotted by pressure-blot on a nitrocellulose membrane.and processed as in Fig. 2B. Lane 1: Reference plasma (ORM phenotype ORM1 F1S-ORM2A) used for the fractionation. Lane 2: Small peak eluted just before peak 1; Lane 3: Peak 1; Lane 4: Peak 2.

two different gene products. Analysis of genomic clones confirmed this hypothesis and revealed that the ORM locus (on chromosome 9) was in fact duplicated, each DNA sequence coding for one isoprotein [7].

In parallel, studies conducted on the analysis of ORM phenotypes demonstrated that the two proteins, produced by the ORMA and B genes, could be separated on IEF–immunoblot after desialylation and were present in (about) every individual [29]. The A locus shows extensive polymorphism, when variants are rarely found at the B locus [8]. The basis for the polymorphism of the A variants, point amino acid substitutions, has been recently identified for the common allele products [30].

Additional complexity comes from the discovery of duplicated alleles at both loci. In their original description of the ORM locus, Dente et al. reported the presence of an additional ORM gene duplicated from the B gene, called B′ and which apparently was not expressed [7]. Further studies reported reduplications at both the A and B loci [31], but recent work demonstrated that the duplication of the A locus is by far the most frequent and present on 20% of the chromosomes in the population studied [32]. The existence of this frequent triplication of the gene, which apparently occurs by unequal recombination, might have been favored by the presence of a repeated pentanucleotide sequence. By contrast, deleted alleles, which should be present at an identical frequency, are very rare, and the only null allele explored was caused not by a deletion, but by a frameshift [32].

Binding differences are observed between the two proteins, which can be of major clinical importance for tightly bound drugs [20,22,24–26,33–35]. Detailed studies measuring displacement of high-affinity drugs from each variant allowed the construction of a three-dimensional model for the ORM2, but not the ORM1 protein [23].

Finally, in addition to polymorphism, ORM exhibits a microheterogeneity, which has been related to differences in the sugar chains of the protein [3,36,37]. Indeed, ORM possesses five N-glycans that belong to the bi-, tri- or tetra-antennary chain group [38–40]. The ratio between these chains is influenced by a series of factors, including pregnancy, estrogen therapy and the acute phase process [41,42]. Among these structures, the sialyl Lewis X motif is thought to be responsible for at least part of the role of ORM as an immunomodulator, through its lectin-like binding properties to various cell receptors [41,43].

In the present article, we report a one-step technique, based upon pseudo-ligand affinity chromatography on immobilized Cibacron Blue, which allows purification of ORM proteins together with their separation according to their genetic origin. Fractionation of proteins on Cibacron Blue is widely used as a first step technique for protein purification [44,45], including that of ORM [3,46,47]. Previous work had shown that the mixture of ORMA and B proteins could be fractionated by an additional step of metal ion affinity chromatography using copper-chelate columns [48,49]. We have shown previously that, at acidic pH, the first protein to be eluted from the Cibacron Blue column was ORM [50]. In the present work, we have determined the parameters necessary to successively separate ORMB and ORMA gene products. It is important to note that the size of the gel plays an important role in the above separation, and that we have been unable to obtain similar results when using the 150−300 mesh gel.

The availability of a simple technique to separate the two ORM gene products should help to determine accurately the respective affinity constants of the ORM1 and 2 proteins (as well as their variants) for basic drugs. It is well established that, for the drugs which bind to ORM proteins, the affinity of this binding may affect drastically their volume of distribution as well as their delivery to target cells [22,26,46]. This, added to the fact that ORM is a major acute-phase reactant [3], whose levels can raise up to five times during pathological conditions, stresses the importance of the determination of ORM parameters in the pharmacokinetics of a number of drugs under development. In this respect, work is in progress in our laboratory to study these interactions using purified allele products immobilized on surface plasmon resonance sensor chips.

Acknowledgements

This work was supported in part by a grant from the National Institute of Drug Abuse RO1-13027-01, and by a grant from Labcatal Laboratories, Montrouge, France. The technical assistance of Catherine

Dopson and Evette Robinson is acknowledged. We thank Biorad Laboratories for a generous gift of Affigel Blue.

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